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Bex1, a novel interactor of the p75 neurotrophin receptor, links neurotrophin signaling to the cell cycle

Marçal Vilar[1,3,4], Maribel Murillo-Carretero[1,3], Helena Mira[2,5], Kalle Magnusson[1], Valerie Besset[1,6] and Carlos F Ibáñez[1,*]

[1]Division of Molecular Neurobiology, Department of Neuroscience, Stockholm, Sweden and [2]Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden

A screening for intracellular interactors of the p75 neurotrophin receptor ($p75^{NTR}$) identified brain-expressed X-linked 1 (Bex1), a small adaptor-like protein of unknown function. Bex1 levels oscillated during the cell cycle, and preventing the normal cycling and downregulation of Bex1 in PC12 cells sustained cell proliferation under conditions of growth arrest, and inhibited neuronal differentiation in response to nerve growth factor (NGF). Neuronal differentiation of precursors isolated from the brain subventricular zone was also reduced by ectopic Bex1. In PC12 cells, Bex1 overexpression inhibited the induction of NF-κB activity by NGF without affecting activation of Erk1/2 and AKT, while Bex1 knockdown accelerated neuronal differentiation and potentiated NF-κB activity in response to NGF. Bex1 competed with RIP2 for binding to the $p75^{NTR}$ intracellular domain, and elevating RIP2 levels restored the ability of cells overexpressing Bex1 to differentiate in response to NGF. Together, these data establish Bex1 as a novel link between neurotrophin signaling, the cell cycle, and neuronal differentiation, and suggest that Bex1 may function by coordinating internal cellular states with the ability of cells to respond to external signals.

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Introduction

Nerve growth factor (NGF) and other members of the neurotrophin family mediate survival, growth and differentiation of neuronal and glial cells by binding to two different types of cell surface receptors, the Trk tyrosine kinases—TrkA, TrkB and TrkC—and the p75 neurotrophin receptor ($p75^{NTR}$). $p75^{NTR}$ resembles other members of the tumor necrosis factor receptor superfamily in the organization of its extracellular domain and in the presence of a small globular domain in the intracellular region, the so-called death domain (Liepinsh *et al*, 1997). $p75^{NTR}$ signaling can contribute to neurotrophin-mediated survival, differentiation and neurite outgrowth in a variety of neuronal subpopulations (Roux and Barker, 2002). On the other hand, $p75^{NTR}$ can also mediate cell death by neurotrophins, proneurotrophins and various amyloid peptides, as well as inhibit axonal growth and regeneration in its capacity of signaling receptor for myelin-inhibitory components (Teng and Hempstead, 2004). Thus, the physiological consequences of $p75^{NTR}$ signaling depend on the cellular context and the nature of the activating ligands (Bronfman and Fainzilber, 2004). Mice lacking $p75^{NTR}$ have a multifaceted phenotype, characterized by both positive and negative effects (Lee *et al*, 1992; von Schack *et al*, 2001; Naumann *et al*, 2002), reflecting the complexity of $p75^{NTR}$ signaling.

Trk receptors transmit intracellular signals through several of the canonical pathways activated by other receptor tyrosine kinases, including the Ras/MAP kinase and phosphatidylinositol-3 kinase (PI3K)/AKT signaling pathways (Kaplan and Miller, 2000; Huang and Reichardt, 2003). In contrast, the signaling mechanisms used by $p75^{NTR}$ have remained elusive. Lacking intrinsic catalytic activity, $p75^{NTR}$ signaling is dependent on the ability of this receptor to interact with components of intracellular signaling pathways. Several different $p75^{NTR}$ interacting molecules, with and without catalytic activity, have been identified to date (Gentry *et al*, 2004). Noncatalytic interactors include a series of scaffolding- and adaptor-like molecules, such as caveolin-1 (Bilderback *et al*, 1997), Bex3/NADE (Mukai *et al*, 2000) and TRAF6 (Khursigara *et al*, 1999; Ye *et al*, 1999); larger proteins containing zinc-finger domains with some degree of nuclear localization, such as NRIF1/2 (Casademunt *et al*, 1999) and SC-1 (Chittka and Chao, 1999); and members of the MAGE homology domain family, such as NRAGE (Salehi *et al*, 2000) and necdin (Tcherpakov *et al*, 2002), with proposed roles in the regulation of apoptosis. $p75^{NTR}$ interactors with catalytic activity include serine–threonine kinases involved in interleukin and NF-κB signaling, such as IRAK (Mamidipudi *et al*, 2002) and RIP2 (Khursigara *et al*, 2001); a protein tyrosine phosphatase (FAP-1) (Irie *et al*, 1999); and the small GTPase RhoA (Yamashita *et al*, 1999). How these $p75^{NTR}$-interacting proteins connect to downstream signaling pathways and cellular responses is less clear, however. Some of the principal downstream events characterized in $p75^{NTR}$ signaling include ceramide production (Dobrowsky *et al*, 1994), and activation of the transcription factor NF-κB (Carter *et al*,

*Corresponding author. Division of Molecular Neurobiology, Department of Neuroscience, Karolinska Institute, Berzelius väg 35, Box 285, Stockholm 17177, Sweden. Tel.: +46 8 524 87660; Fax: +46 8 33 9548; E-mail: carlos.ibanez@neuro.ki.se
[3]These authors contributed equally to this work
[4]Present address: Structural Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA, USA
[5]Present address: Departamento de Biologia Celular, Universitat de Valencia, Spain
[6]Present address: Sanofi-Aventis, 94400 Vitry sur Seine, France

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1996) and the c-Jun kinases JNK1–3 (Casaccia-Bonnefil *et al*, 1996; Friedman, 2000; Harrington *et al*, 2002).

The rat pheochromocytoma cell line PC12 expresses both TrkA and $p75^{NTR}$ and has become a principal model to study NGF action. In response to NGF, PC12 cells exit the cell cycle, extend neurites and differentiate into a neuronal cell type that resembles sympathetic neurons (Greene and Tischler, 1976). An extensive body of evidence supports the fundamental role of TrkA signaling in PC12 cell differentiation in response to NGF (Huang and Reichardt, 2003). More recently, the contribution of $p75^{NTR}$ to this process has also begun to be appreciated (Foehr *et al*, 2000; Wooten *et al*, 2001; Hosomi *et al*, 2003; Sole *et al*, 2004). NGF signaling in PC12 cells is regulated by the cell cycle, leading to cell differentiation during G1 but to cell cycle progression during other cycle phases (Rudkin *et al*, 1989). NGF causes an accumulation of cells in the G1 phase as a result of a block in the transition from G1 to S phase (van Grunsven *et al*, 1996). Interestingly, NGF receptors present a cyclical expression at the extracellular surface of exponentially growing PC12 cells, with high levels of TrkA during M and early G1, and of $p75^{NTR}$ during late G1, S and G2 (Urdiales *et al*, 1998). The molecular mechanisms linking NGF receptor signaling to the cell cycle, however, remain poorly understood (Lopez-Sanchez and Frade, 2002).

In the present study, we set out to identify novel intracellular interactors of $p75^{NTR}$ using a functional screening method based on T7 phage display, and identified the brain-expressed X-linked 1 (Bex1) protein as a new intracellular interactor of $p75^{NTR}$. Our results indicate that Bex1 may represent a distinct class of upstream intracellular modulators of neurotrophin receptors, linking neurotrophin signaling to the cell cycle.

Results

Identification of Bex1 as an intracellular interactor of $p75^{NTR}$

A C-terminal fragment of the rat ortholog of a human protein originally named Bex1 (Brown and Kay, 1999) was isolated in a T7 phage display screen for interacting partners of the intracellular domain of $p75^{NTR}$ (Figure 1A). Up to six paralog *Bex* genes have been identified in rodents and humans (Alvarez *et al*, 2005; Koo *et al*, 2005). Bex1 and Bex2 are very similar in protein sequence (87% identity), while Bex3/NADE—a previously characterized interactor of the $p75^{NTR}$ death domain (Mukai *et al*, 2000)—is only 30% identical to either Bex1 or Bex2 and thus represents a more divergent member of this family (Figure 1A). Despite their low similarity, the main region in Bex3/NADE implicated in its interaction with $p75^{NTR}$ was included in the Bex1 cDNA fragment isolated by phage display (Figure 1A), suggesting the existence of a conserved $p75^{NTR}$-binding interface among Bex proteins.

$p75^{NTR}$ could be specifically co-immunoprecipitated with hemagglutinin (HA)-tagged Bex1 in transfected HEK293 T (293) cells (Figure 1B). Flag-tagged Bex1 (Flag-Bex1) could also be co-immunoprecipitated with endogenous $p75^{NTR}$ in two PC12 cell clones—termed E2 and C4—generated by stable transfection (Figure 1C and D). Using anti-Bex1 anti-

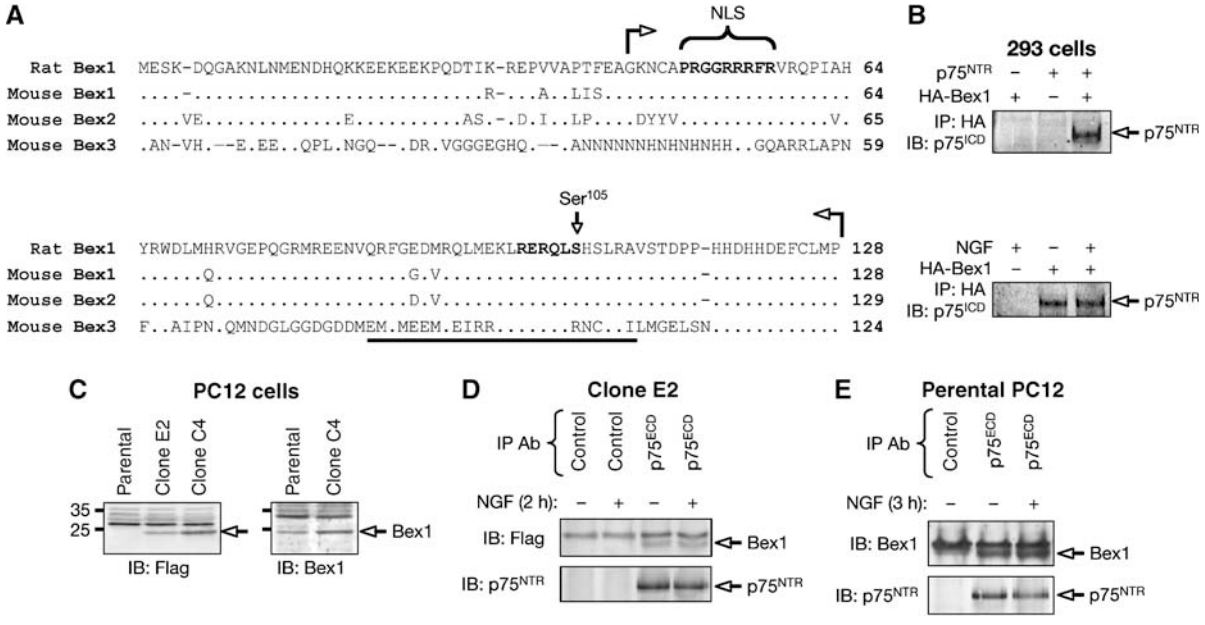

Figure 1 Identification of Bex1 as a novel intracellular interactor of $p75^{NTR}$. (**A**) Alignment of primary amino-acid sequences of rat Bex1 and mouse Bex1, Bex2 and Bex3 performed with ClustalX (Jeanmougin *et al*, 1998). The region of rat Bex1 identified by T7 phage display is indicated between arrows. The $p75^{NTR}$-binding site in Bex3/NADE is underlined (Mukai *et al*, 2000). NLS and Ser^{105} are indicated. (**B**) Interaction between HA-tagged Bex1 (HA-Bex1) and $p75^{NTR}$ overexpressed in 293 cells analyzed by IP with anti-HA antibodies and Western IB with antibodies against the intracellular domain of $p75^{NTR}$ ($p75^{ICD}$). In the lower panel, transfected cells were treated for 12 h with NGF prior to lysis and IP as indicated. (**C**) IB of cell lysates (50 μg protein) from parental PC12 cells and clones C4 and E2 overexpressing Flag-Bex1 using anti-Flag (left) or anti-Bex1 antibodies. (**D**) Interaction between endogenous $p75^{NTR}$ and Flag-Bex1 in clone E2 in the presence or absence of NGF analyzed by IP with control antibodies or antibodies against the extracellular domain of $p75^{NTR}$ ($p75^{ECD}$), followed by IB with anti-Flag antibodies. (**E**) Interaction between endogenous Bex1 and $p75^{NTR}$ in parental PC12 cells analyzed by IP with control or anti-$p75^{ECD}$ antibodies, followed by IB with anti-Bex1 antibodies.

bodies, endogenous Bex1 could be specifically co-immunoprecipitated with endogenous $p75^{NTR}$ in parental PC12 cells (Figure 1E), indicating that the two proteins are able to interact when expressed at physiological levels. Association between endogenous Bex1 and $p75^{NTR}$ was also observed in lysates of primary Schwann cells (data not shown). NGF treatment did not affect the interaction between Bex1 and $p75^{NTR}$—either transfected or endogenous (Figure 1B, D and E).

Overlapping expression of Bex1 and $p75^{NTR}$ mRNAs in developing mesenchyme and vascular and nervous tissues

Expression of *Bex1*, *Bex2* and *Bex3* mRNAs could be detected by RT–PCR in PC12 cells and primary cultures of embryonic day (E) 14.5 rat dorsal root ganglion (DRG) cells (Figure 2A). Only *Bex1* and *Bex3* mRNAs could be found in Schwann cells isolated from the newborn rat sciatic nerve and in E17.5 hippocampal cultures (Figure 2A). mRNAs encoding all three Bex isoforms could also be detected in newborn (P1) cerebral cortex, hippocampus and olfactory bulb (Figure 2B). *Bex1* mRNA expression could be localized more precisely by *in situ* hybridization within specific regions of the developing rat embryo (Figure 2C). At E13.5, *Bex1* mRNA was widely expressed throughout the developing nervous system and in vascular and mesenchymal structures. In particular, prominent *Bex1* mRNA expression could be observed in the somitic mesenchyme, heart, aorta, dorsal root ganglia, sympathetic ganglia and the lateral motor columns of the developing spinal cord, all tissues known to express high levels of $p75^{NTR}$ mRNA at this stage (Ernfors *et al*, 1988; Huber and Chao, 1995; Cotrina *et al*, 2000; von Schack *et al*, 2001). At later stages of brain development, *Bex1* mRNA was also observed in structures known to express $p75^{NTR}$ mRNA, including the olfactory bulb, striatum, thalamus, cerebral cortex and hippocampus (Figure 2C). In the cortex, *Bex1* mRNA was detected in the subplate and cortical plate, where cells expressing $p75^{NTR}$ mRNA are known to reside at this stage (DeFreitas *et al*, 2001; Kendall *et al*, 2003).

Dynamic nucleocytoplasmic trafficking of Bex1 in response to NGF in $p75^{NTR}$-expressing cells

The subcellular localization of Bex1 was visualized by introducing a Bex1 construct tagged with a green fluorescent

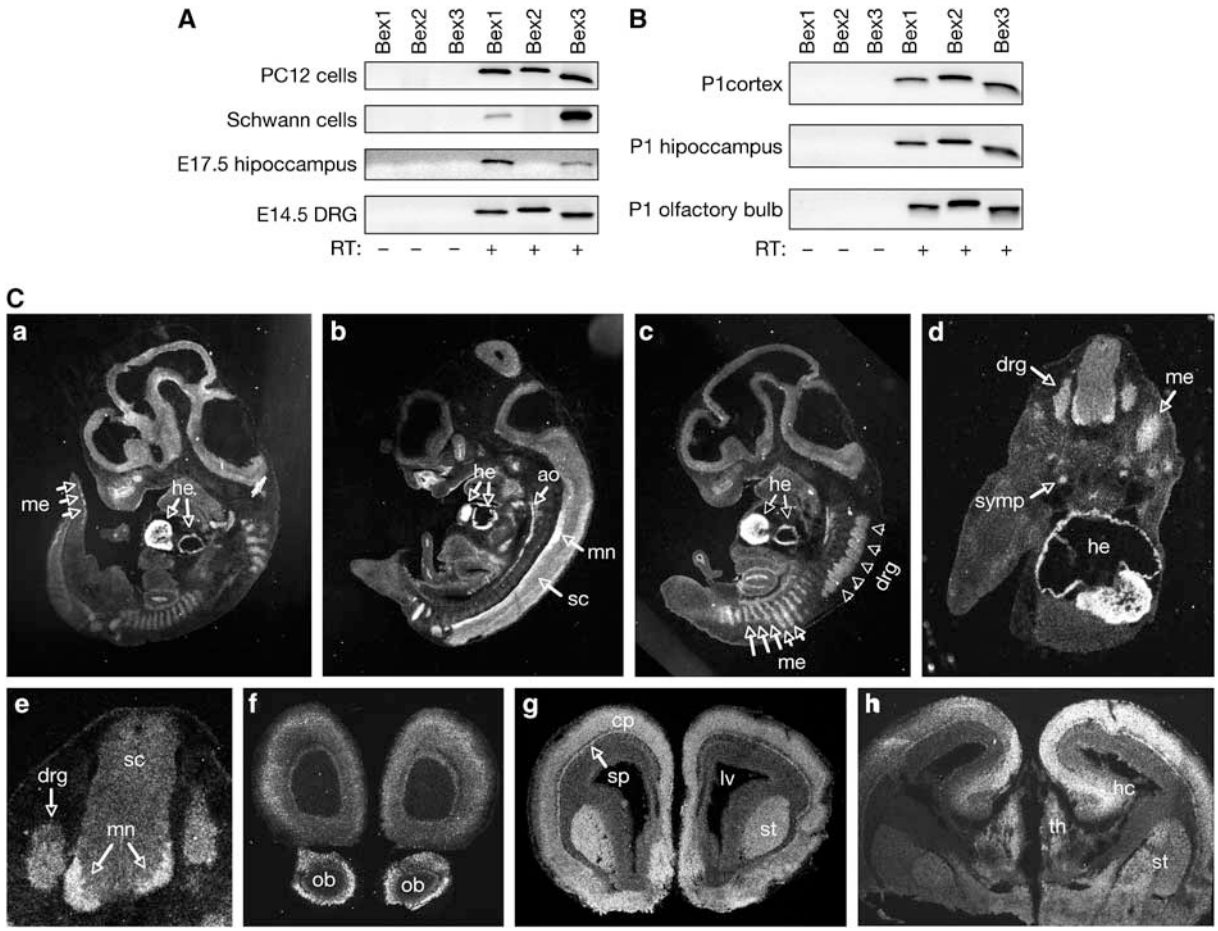

Figure 2 Overlapping expression of *Bex1* and $p75^{NTR}$ mRNAs in developing mesenchyme and vascular and nervous tissues. (**A**) RT–PCR analysis of *Bex1*, *Bex2* and *Bex3* mRNA expression in cultures of PC12 cells, Schwann cells, E17.5 hippocampus and E14.5 dorsal root ganglia (DRG). RT, reverse transcriptase. (**B**) RT–PCR analysis of *Bex1*, *Bex2* and *Bex3* mRNA expression in P1 cerebral cortex, hippocampus and olfactory bulb. (**C**) *Bex1* mRNA expression analyzed by *in situ* hybridization in sagittal (a–c) and transverse (d, e) sections of E13.5 rat embryos, and in coronal sections of E19.5 rat brain (f–h). me, mesenchyme; he, heart; ao, aorta; sc, spinal cord; mn, motorneurons; drg, dorsal root ganglia; symp, sympathetic ganglia; ob, olfactory bulb; st, striatum; cp, cortical plate; sp, subplate; lv, lateral ventricle; hc hippocampus; th, thalamus.

protein moiety (GFP-Bex1) into 293, PC12 and Schwann cells. GFP fluorescence was present in both nucleus and cytoplasm of all these cell types, with 80–85% of cells displaying brighter fluorescence in the nucleus, and 15–20% in the cytoplasm (Figure 3A, B and G). A putative nuclear localization signal (NLS) was identified in the N-terminal half of Bex1 (Figure 1A). Mutation of the Arg^{53}–Arg^{54}–Arg^{55} triplet in this motif into Ala–Ala–Ala (GFP-$Bex1^{mutNLS}$) excluded GFP-Bex1 from the nucleus of 293 cells (Figure 3A), indicating that Bex1 harbors a functional NLS. In cells cotransfected with Bex1 and $p75^{NTR}$, NGF produced a redistribution of GFP-Bex1 from the nucleus to the cytoplasm (Figure 3A), suggesting that NGF may be able to regulate the nuclear export of Bex1 in cells expressing $p75^{NTR}$.

Cell fractionation and immunoblotting (IB) showed that 60–70% of all GFP-Bex1 was present in the cytoplasm of transiently transfected PC12 cells (Figure 3B). Similar proportions were also observed in stably transfected (Figure 3C) and parental PC12 cells (Figure 3D), showing that both ectopic and endogenous Bex1 have a similar subcellular distribution. The apparent discrepancy between IB and GFP fluorescence is likely due to the compact size and shape of the nucleus, which makes the fluorescence signal appear to be more concentrated in this compartment (e.g. Figure 3B). The proportion of PC12 cells that showed a higher concentration of GFP-Bex1 fluorescence in the cytoplasm—normally 15–20%—increased over time upon NGF treatment to 30–35% (Figure 3E), indicating the ability of NGF to regulate Bex1 translocation. NGF had no effect in PC12 cell lines that lack expression of full-length $p75^{NTR}$, such as mR-1 cells (Benedetti *et al*, 1993) (Figure 3E) or NRA5 cells (Rabizadeh *et al*, 1994) (data not shown), suggesting a role for $p75^{NTR}$ in the regulation of Bex1 nucleocytoplasmic trafficking. In agreement with this, BDNF—which can interact with $p75^{NTR}$ but not TrkA—was also able to increase the proportion of PC12 cells showing GFP-Bex1 predominantly in the cytoplasm (Figure 3F). Similar to PC12 cells, nuclear GFP-Bex1 relocalized to the cytoplasm in 30% of Schwann cells within 60 min of NGF treatment (Figure 3G and H). Since Schwann cells do not express TrkA, these results indicated

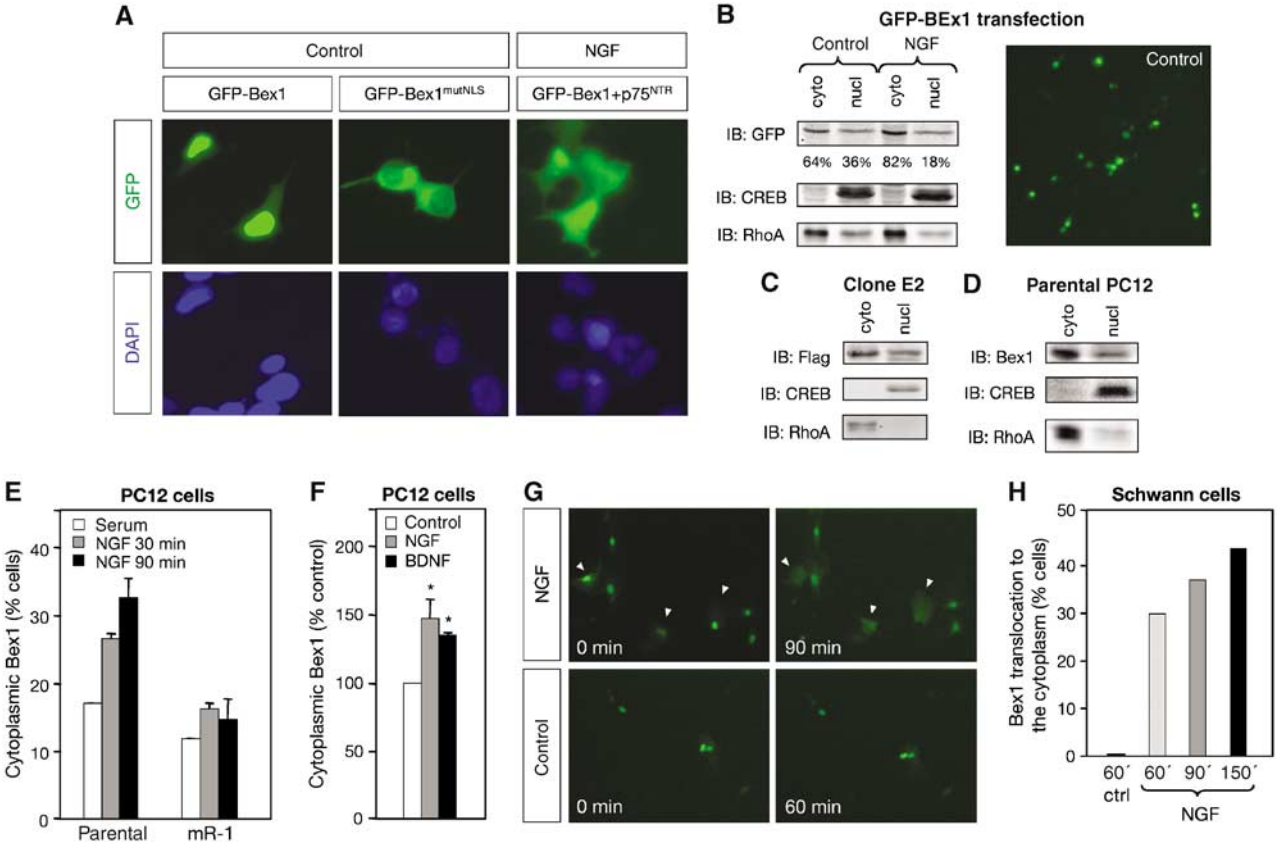

Figure 3 Dynamic nucleocytoplasmic trafficking of Bex1 in response to NGF. (**A**) 293 cells were transfected with GFP-Bex1, GFP-$Bex1^{mutNLS}$ or GFP-Bex1 and $p75^{NTR}$, and treated with NGF for 2 h as indicated. (**B**) Subcellular localization of GFP-Bex1 in transiently transfected PC12 cells—before or after 90 min with NGF—was assessed by fractionation and IB with GFP antibodies. CREB and RhoA were used as markers for nuclear and cytoplasmic compartments, respectively. Percentage in each fraction is indicated. A micrograph of cells expressing GFP-Bex1 grown under control conditions is shown to the right. (**C**) Subcellular localization of Flag-Bex1 in stable transfected PC12 cells (clone E2). (**D**) Subcellular localization of endogenous Bex1 in naïve PC12 cells. (**E**) GFP-Bex1 was transiently transfected in parental or mR-1 PC12 cells and the percentage of cells displaying GFP-Bex1 in a predominant cytoplasmic localization following NGF treatment during the indicated times was quantified. A minimum of 900 cells were counted per condition. Results are average of three independent experiments ± s.e.m. (**F**) Translocation of GFP-Bex1 after NGF or BDNF treatment in PC12 cells. $^*P<0.05$ versus control. (**G**) GFP-Bex1 was transiently transfected in Schwann cells and photographed at different times after NGF or control treatments. Arrowheads indicate cells showing cytoplasmic translocation of Bex1 in response to NGF treatment. (**H**) The number of Schwann cells showing translocation of Bex1 from the nucleus to the cytoplasm after the indicated treatments and times was quantified. Results are percentage of cells showing Bex1 translocation at each time point. In all, 70 cells were observed in each case.

that nucleocytoplasmic trafficking of Bex1 in response to NGF must have been mediated by $p75^{NTR}$ signaling.

Dynamic regulation of Bex1 protein levels during the cell cycle and upon neuronal differentiation

Prolonged serum withdrawal—which arrests PC12 cells in the G1 phase of the cell cycle (Rudkin *et al*, 1989; Urdiales *et al*, 1998)—drastically affected the endogenous levels of Bex1 protein in PC12 cells, with little or no Bex1 detected after 24 h in serum-free medium (Figure 4A). Bex1 expression recovered in arrested cells after prolonged NGF treatment and concomitantly with postmitotic neuronal differentiation (Figure 4A), in agreement with the abundant Bex1 expression observed in postmitotic neurons *in vivo* (Figure 2C). In order to examine the regulation of Bex1 levels during the cycle, cell cultures were synchronized in G1 by 24 h serum starvation, after which serum was added back to initiate cell cycle re-entry. Bex1 levels were largely restored 12 h after serum re-addition, as the cells entered S phase, and then decreased again 36–48 h thereafter, as cells returned to G1 (Figure 4B). A second peak in Bex1 was observed as cells re-entered S phase 72 h after serum addition (Figure 4B). Thus, the levels of endogenous Bex1 oscillated during the cell cycle in a pattern that resembled that of $p75^{NTR}$ expression in the membrane of PC12 cells, which had previously been found to be maximal during S phase and lowest during early G1 (Urdiales *et al*, 1998).

Serum starvation of PC12 cells did not affect the steady-state level of *Bex1* mRNA (Figure 4C), indicating a post-transcriptional regulation of Bex1 levels. Using the protein synthesis inhibitor cycloheximide, the half-life of Bex1 was estimated at approximately 5 h in exponentially growing cells (Figure 4D). Upon serum withdrawal, however, Bex1 declined even more rapidly, falling below detection levels between 30 and 90 min after serum deprivation (Figure 4E and data not shown). Interestingly, this decline could be prevented by treatment with the proteasome inhibitor PS I (Figure 4E), confirming that Bex1 levels are regulated by protein degradation.

Phosphorylation of Bex1 in Ser^{105} by the serine–threonine kinase AKT stabilizes Bex1 and protects it from proteasomal degradation

A motif in the C-terminal portion of the protein (^{100}RERQLS^{105}) matched the consensus site for phosphorylation by the serine–threonine kinase AKT (RxRxxS/T) (Figure 5A). After metabolic labeling of 293 cells with ^{32}P-orthophosphate, incorporation of ^{32}P was observed in wild-type Bex1, but not in a mutant with a Ser^{105} to Ala substitution (Figure 5B), indicating that Ser^{105} is an endogenous phosphorylation site in this protein. In addition, no phosphorylated Bex1 could be detected after treatment with LY294002, an inhibitor of PI3K, the upstream activator of AKT (Figure 5C). Moreover, reduction of endogenous AKT activity by serum withdrawal also attenuated Bex1 phosphorylation (Figure 5C). In *in vitro* kinase assays, wild type, but not mutant, Bex1 was readily phosphorylated by wild-type AKT, while kinase-dead AKT had no effect (Figure 5D), indicating that Bex1 is a direct target of AKT *in vitro*. Using antibodies that recognize a phosphorylated consensus substrate site for AKT, an increase in the phosphorylation of a band corresponding to Bex1 could be observed in PC12 cells treated with NGF, a stimulus that is known to acutely activate AKT in these cells (Figure 5E). $Bex1^{S105A}$ was consistently expressed at levels lower than wild-type Bex1 in exponentially growing cells (e.g. Figure 5B and F), suggesting a role for Ser^{105} phosphorylation in regulating the stability of the protein. In agreement with this, treatment with the proteaso-

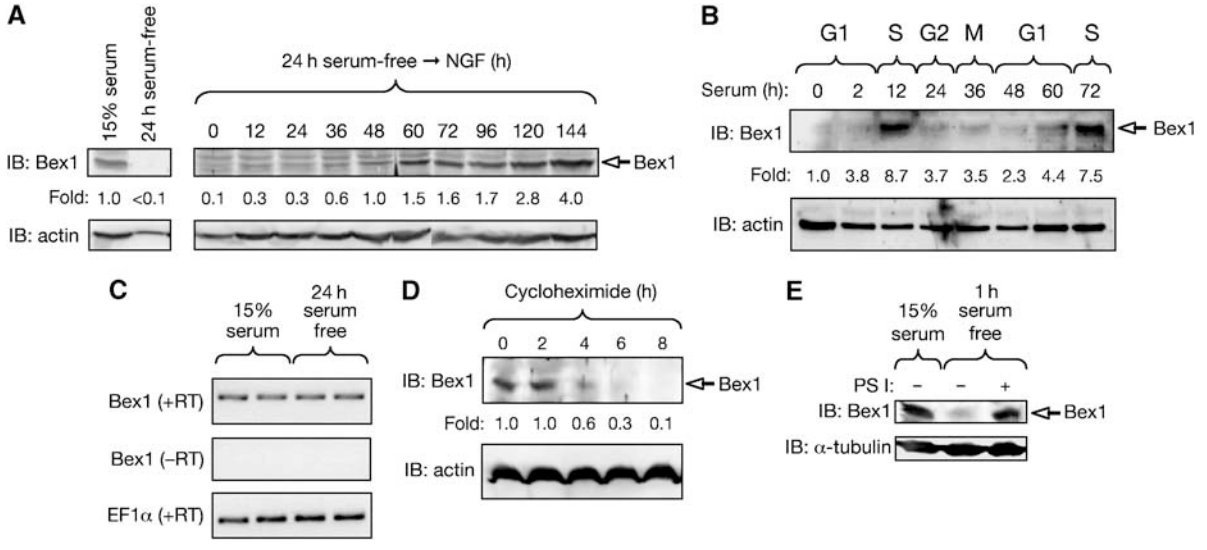

Figure 4 Dynamic regulation of Bex1 protein levels during the cell cycle and upon neuronal differentiation. (**A**) PC12 cell cultures were serum-starved for 24 h and then treated with NGF plus 1.5% serum for up to 6 days. Expression of endogenous Bex1 was analyzed by IB. Re-probing for actin was used as loading control. Fold change relative to actin is indicated. Similar results were obtained in three independent experiments. (**B**) PC12 cell cultures were serum-starved for 24 h and then re-exposed to serum for up to 3 days. Expression of endogenous Bex1 was analyzed by IB. Fold change relative to actin is indicated. Similar results were obtained in three independent experiments. (**C**) RT–PCR analysis of *Bex1* mRNA levels in exponentially growing and serum-starved PC12 cells. RT–PCR for EF-1α was used as loading control. (**D**) Exponentially growing cultures of PC12 cells were treated with cycloheximide for the indicated periods of time and subsequently analyzed for endogenous expression of Bex1 by IB. (**E**) Endogenous Bex1 was analyzed by IB in PC12 cells after 1 h serum-starving in the presence or absence of PS I. Levels of Bex1 in exponentially growing cells (15% serum) are shown as control. Reprobing for tubulin was used as loading control.

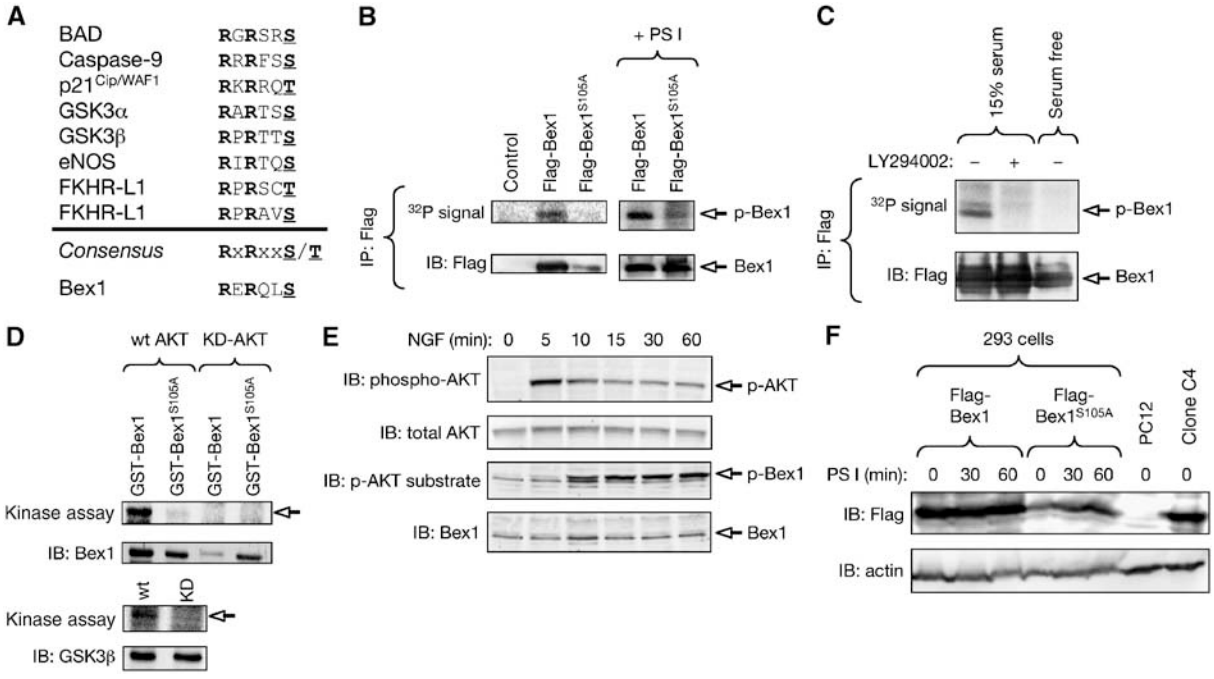

Figure 5 Phosphorylation of Bex1 in Ser^{105} by the serine–threonine kinase AKT. (**A**) Consensus sites for phosphorylation in a subset of known AKT substrates shown together with the corresponding site in the Bex1 sequence. (**B**) Analysis of ^{32}P incorporation in HEK293 T cells that had been metabolically labeled with ^{32}P-orthophosphate after transfection with Flag-tagged versions of wild-type Bex1 or a mutant Bex1 with a Ser^{105} to Ala substitution (Flag-Bex1^{S105A}). An autoradiogram of anti-Flag immunoprecipitates and its corresponding control IB with anti-Flag antibodies is shown. The panel to the right shows the same experiment performed in cells treated with the proteasomal inhibitor PS I, showing that the reduced phosphorylation of the S105A mutant was not due to its otherwise low level of expression. (**C**) Bex1 phosphorylation analyzed by metabolic labeling of transfected HEK293 T cells following 2 h treatment with the PI3K inhibitor LY294002 or after 24 h serum withdrawal. (**D**) Kinase assay of bacterially produced GST-tagged Bex1 constructs incubated with lysates of HEK293 T cells transiently transfected with wild-type or kinase-dead versions of AKT. An autoradiogram of GST pull-downs and its corresponding control IB with anti-GST antibodies is shown. GSK3β was used as positive control (lower panels). Arrows denote phosphorylated products. (**E**) Analysis of AKT activation and Bex1 phosphorylation in PC12 cells treated with NGF. Immunoblots of phospho-AKT, total AKT, phospho-AKT substrates and Bex1 are shown. Phospho-AKT and phospho-Bex1 are indicated. (**F**) Steady-state levels of Flag-tagged wild type and S105A Bex1 in HEK293 T cells in the presence and absence of the proteasomal inhibitor PS I analyzed by IB with anti-Flag antibodies. Lysates of parental and clone C4 PC12 cells were used as negative and positive controls, respectively.

mal inhibitor PS I produced a significant increase in the levels of Bex1^{S105A} (Figure 5F). Using PS I, we could also confirm that the reduced phosphorylation of the S105A mutant observed in 293 cells was not due to its otherwise low level of expression (Figure 5B, right panel). Taken together, these results indicated that Bex1 can be phosphorylated in Ser^{105} by AKT, and that this event regulates Bex1 protein turnover.

Deregulated Bex1 expression prevents growth arrest and neuronal differentiation of PC12 cells

We could not observe any significant increase in cell death after transient transfection of GFP-Bex1 in PC12 cells (Supplementary Figure 1), nor in stable PC12 clones overexpressing Bex1 (data not shown). Parental PC12 cells and clones overexpressing Bex1 showed no differences in cell cycle progression during exponential growth (Figure 6A and data not shown). However, upon 24 h serum withdrawal, a larger proportion of cells in clones C4 and E2 were in S phase compared to parental cells (Figure 6A and Supplementary Figure 2), suggesting that Bex1 overexpression may have interfered with cell cycle arrest. Incorporation of BrdU was examined under two growth-arrest conditions, that is, serum withdrawal and NGF treatment. Strikingly, cells overexpressing Bex1 continued proliferating at normal levels under either condition (Figure 6B), indicating a failure to exit the cell cycle as a result of deregulated Bex1 expression. Following 5-day treatment with NGF in low serum, clones overexpressing Bex1—as well as transiently transfected cells—failed to differentiate, and showed only incipient signs of neuronal differentiation (Figure 6C and data not shown). Taken together, these results indicated a role for Bex1 in cell cycle progression and neuronal differentiation.

Bex1 modulates proximal signaling by p75^{NTR}, but not TrkA

Although PC12 clones overexpressing Bex1 were refractory to NGF-induced growth arrest and differentiation, they showed survival responses to this factor that were comparable to those of parental PC12 cells (Figure 6D). In agreement with this, NGF stimulation of parental PC12 cells or clones overexpressing Bex1 resulted in comparable activation of the Ras/MAPK and the PI3K/AKT pathways, the two major signaling pathways activated downstream of TrkA (Figure 7A), suggesting that Bex1 overexpression did not interfere with proximal TrkA signaling.

The first pathway to be characterized downstream of p75^{NTR} leads to activation of the NF-κB transcription factor (Carter *et al*, 1996), and has been implicated in survival (Wood, 1995; Yoon *et al*, 1998; Hamanoue *et al*, 1999) and neuronal differentiation (Foehr *et al*, 2000; Wooten *et al*,

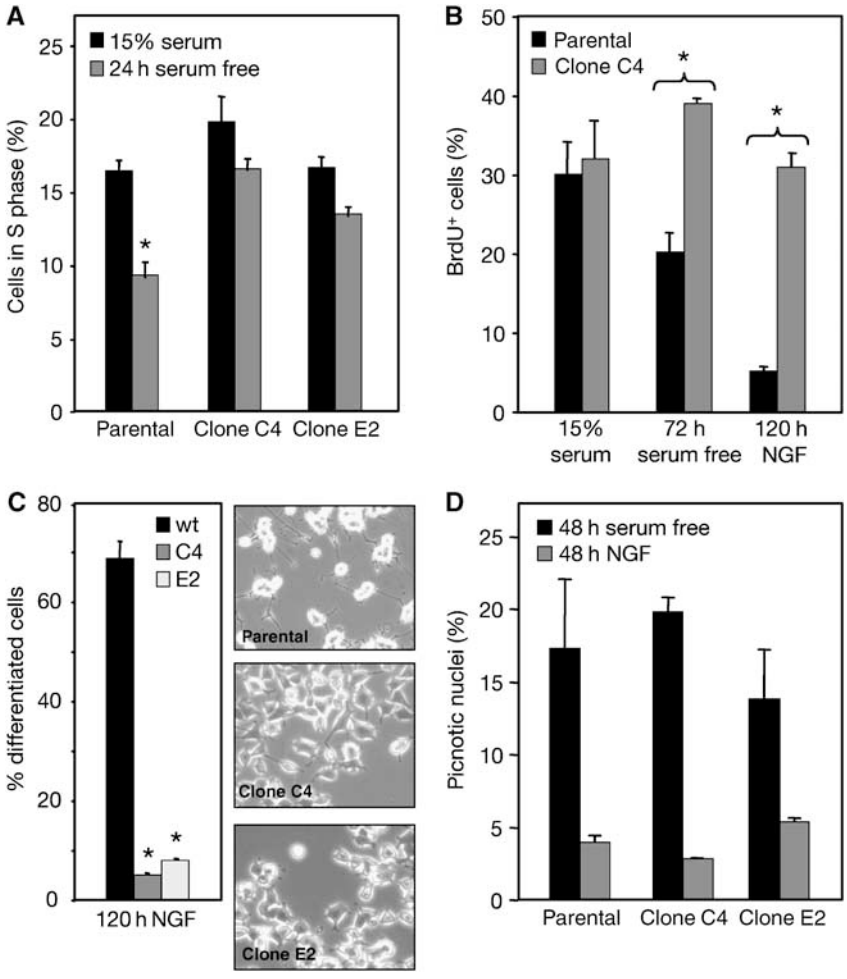

Figure 6 Deregulated Bex1 expression prevents growth arrest and neuronal differentiation of PC12 cells. (**A**) The percentage of cells in S phase was determined by flow cytometry analysis of exponentially growing and serum-starved cultures of parental PC12 and clones C4 and E2 overexpressing Bex1. Results shown are the mean ± s.e.m. of three independent experiments, each performed in triplicate. *$P<0.01$ versus 15% serum. (**B**) The percentage of BrdU-positive cells was determined by immunofluorescence analysis of BrdU incorporation in exponentially growing (15% serum), serum-starved and NGF-treated cultures of parental PC12 and clone C4 cells. Counterstaining of nuclei was done with DAPI. Results are shown as average ± s.e.m. of three independent experiments each performed in triplicate. *$P<0.01$. (**C**) The percentage of differentiated cells (i.e. bearing neurites longer than two-cell body diameters) was quantified in parental PC12 and clones C4 and E2 5 days after NGF treatment. Results are shown as average ± s.e.m. of three independent experiments each performed in triplicate. *$P<0.01$ versus parental PC12 cells (wt). Representative micrographs of NGF-treated cultures are shown. (**D**) Percentage of dead cells in parental PC12 cells and clones C4 and E2 after 48 h serum starvation in the presence or absence of NGF. Results are shown as average ± s.e.m. of three independent experiments each performed in triplicate.

2001) of PC12 cells and other cell types. Although similar levels of basal NF-κB activity could be observed in parental cells and clones overexpressing Bex1, NGF was able to stimulate this pathway only in parental cells (Figure 7B), indicating that Bex1 can modulate NF-κB activity in response to NGF in these cells. Although TrkA has also been implicated in the induction of NF-κB activity by NGF in PC12 cells (Foehr *et al*, 2000), the fact that the two most likely candidate pathways for NF-κB activation by TrkA—that is, Ras/MAPK and PI3K/AKT—were unaffected by Bex1 overexpression, suggested interference with $p75^{NTR}$-derived signals.

The serine–threonine kinase RIP2 has been shown to be required for NF-κB activation by $p75^{NTR}$ in response to NGF, and interacts directly with the death domain of this receptor upon NGF stimulation (Khursigara *et al*, 2001). A progressive reduction in the association between RIP2 and $p75^{NTR}$ was observed in cells that received increasing amounts of Bex1 (Figure 7C), suggesting that Bex1 and RIP2 competed for an overlapping binding site in $p75^{NTR}$. In agreement with this, RIP2 transfection in cells overexpressing Bex1 completely rescued neuronal differentiation in response to NGF (Figure 7D).

Bex1 knockdown potentiates NF-κB activity and accelerates PC12 cell differentiation in response to NGF

An siRNA construct directed to the rat *Bex1* mRNA sequence significantly reduced—but did not completely abolish—endogenous *Bex1* mRNA levels (Figure 8A). A small increase in *Bex2* mRNA could also be observed (Figure 8A). Cotransfection of Bex1 siRNA and Flag-Bex1 indicated a reduction of 60–70% in Bex1 protein levels in siRNA-treated cells (Figure 8B). In agreement with a role for Bex1 in the regulation of NF-κB activity, Bex1 knockdown potentiated NF-κB activity and produced a more robust response to NGF (Figure 8C). In addition, a significant potentiation of the differentiation response was observed after 12 h of NGF in

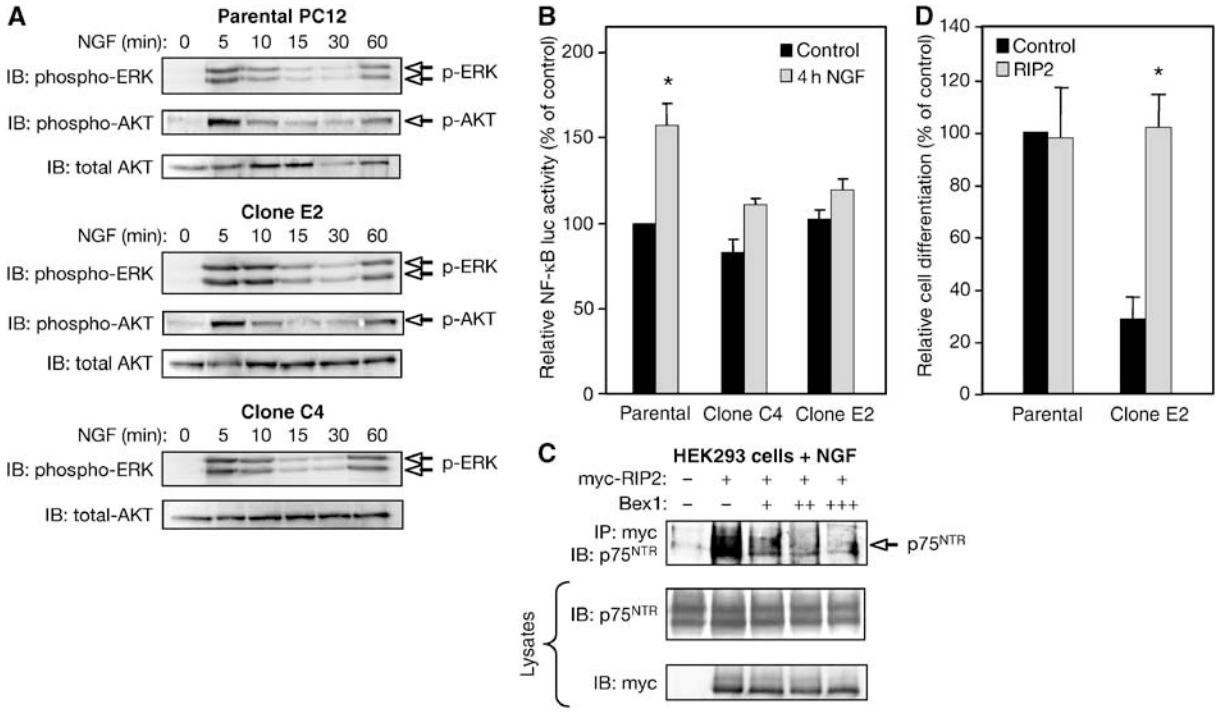

Figure 7 Bex1 modulates proximal signaling by $p75^{NTR}$ but not TrkA. (**A**) NGF-mediated phosphorylation of ERK and AKT was determined in parental PC12 cells and clones overexpressing Bex1 by IB of cell lysates using phospho-specific antibodies. Reprobing of filters with total AKT was used as loading control. (**B**) NF-κB activity was assessed using a firefly luciferase reporter gene in parental PC12 cells and clones overexpressing Bex1 before or after a 4 h treatment with NGF. Luciferase activity was normalized to that of a control construct carrying a Renilla luciferase gene. Results are expressed as average ± s.e.m. of three independent experiments each performed in quadruplicate. $^*P<0.05$ versus control. (**C**) HEK293 T cells were cotransfected with $p75^{NTR}$, myc-tagged RIP2 and increasing amounts of Bex1. After a 4 h treatment with NGF, RIP2 was immunoprecipitated from cell lysates with anti-myc antibodies and filters were probed with anti-$p75^{NTR}$ antibodies. Aliquots of total cell lysates were directly probed with anti-myc and anti-$p75^{NTR}$ antibodies as control. (**D**) RIP2 was transiently transfected in parental PC12 cells and clone E2 together with a GFP expression plasmid and then stimulated with NGF. After 2 days, the fraction of differentiated cells was counted in each condition. Results were normalized to the extent of cell differentiation observed in control parental cells (i.e. 25–32% of transfected cells), and are expressed as average ± s.e.m. of three independent experiments each performed in triplicate. $^*P<0.05$ versus control.

synchronized PC12 cells expressing Bex1 siRNA (Figure 8D, see Supplementary data for details). Interestingly, such levels of differentiation would not normally be observed in naïve cells until after 24–36 h of NGF treatment, that is, roughly the time it takes for cells to move from S to G1 and endogenous Bex1 to disappear. In agreement with this notion, the differentiation advantage of siRNA-treated cells waned over time, such that no difference could be seen between the two groups by 60 h of NGF treatment (Figure 8D).

Bex1 inhibits neuronal differentiation of precursors from the brain subventricular zone

The general ability of Bex1 to regulate neuronal differentiation was tested in neural precursors derived from the brain subventricular zone (SVZ). Proliferating SVZ precursors expressed *Bex1*, *Bex2* and *Bex3* mRNA as assessed by RT–PCR (Figure 9A). Upon differentiation, however, the expression levels of all three genes declined significantly (Figure 9A). We transfected Bex1 into proliferating SVZ precursors which, after an additional round of neurosphere culture, were induced to differentiate by mitogen withdrawal. 9B). Ectopic Bex1 interfered with neuronal differentiation—assessed by coexpression of GFP and βIII-tubulin—of neurosphere-amplified SVZ precursors, as only half as many cells overexpressing Bex1 were able to differentiate into neurons compared to control (Figure 9B and C).

Discussion

The search for intracellular interacting partners of $p75^{NTR}$ has opened multiple inroads into the signaling mechanisms of this receptor. Candidate $p75^{NTR}$ interactors have been identified through either educated guesses (Khursigara *et al*, 1999, 2001; Mamidipudi *et al*, 2002) or the yeast-two-hybrid technique (Casademunt *et al*, 1999; Chittka and Chao, 1999; Yamashita *et al*, 1999; Mukai *et al*, 2000; Salehi *et al*, 2000; Tcherpakov *et al*, 2002). Using T7 phage display, we have identified the Bex1 protein as an intracellular interactor of $p75^{NTR}$. The expression of Bex1 during embryonic development showed a remarkable overlap with that of $p75^{NTR}$ throughout the developing nervous system and in vascular and mesenchymal structures. In transverse sections of E13.5 rat embryos, for example, the predominant pattern of Bex1 mRNA expression—that is, DRG, sympathetic and motorneurons, somitic mesenchyme and heart—was essentially indistinguishable from that of $p75^{NTR}$ at the same developmental stage (Ernfors *et al*, 1988). This is unlike many other intracellular interactors of $p75^{NTR}$ which, for the most part, display a much broader pattern of expression than the receptor (Kendall *et al*, 2002, 2003). The significance of this observation is unclear at present, but given the emerging roles of $p75^{NTR}$ in stem cell renewal and neuronal and glial differentiation, and the ability of Bex1 to modulate similar processes, it suggests that Bex1 may play an important role in

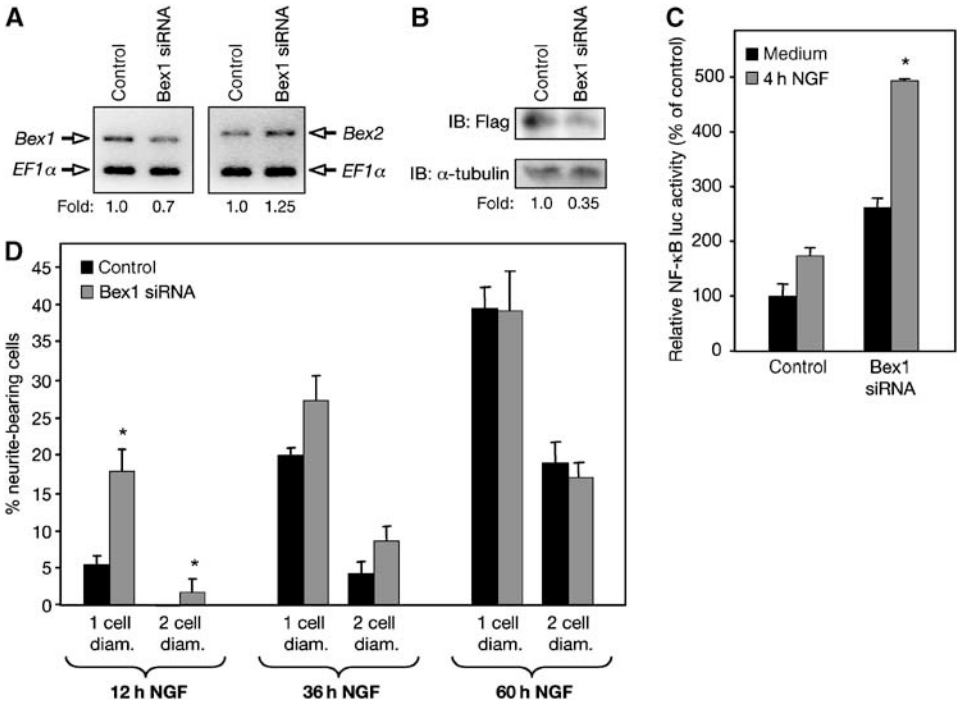

Figure 8 Bex1 knockdown potentiates NF-κB activity and accelerates PC12 cell differentiation in response to NGF. (**A**) *Bex1* and *Bex2* mRNA levels were analyzed by RT–PCR in PC12 cells transfected with a Bex1 siRNA or a control construct. *EF1α* is shown as loading control. Fold changes relative to control are indicated. (**B**) Bex1 protein levels were analyzed by IB in PC12 cells transfected with Bex1 siRNA or a control construct together with Flag-Bex1. α-Tubulin is shown as loading control. Fold change relative to control is indicated. (**C**) The effects of Bex1 knockdown on NF-κB activity were tested in PC12 cells that also received small amounts of ectopic RIP2 (which in our hands enhances NF-κB responses to NGF without altering basal levels), before (medium) or after a 4 h treatment with NGF. Results are expressed as average ± s.e.m. of three experiments performed in quadruplicate. $*P<0.05$ versus control. (**D**) Neuronal differentiation was assessed in synchronized in S phase, PC12 cells transfected with Bex1 siRNA or a control construct as the percentage of cells displaying neurites longer than one or two cell diameters, as indicated (see Supplementary data for details). Results are shown as average ± s.d. of a representative experiment performed in triplicate. $*P<0.05$ versus control. Similar results were obtained in two additional experiments.

coupling $p75^{NTR}$ signaling to the intracellular machinery controlling cell renewal, cell cycle exit and differentiation. The expression of Bex1 in many postmitotic neurons of the peripheral and central nervous systems suggests other functions for this protein—unrelated to cell cycle and growth control—which remain to be explored.

Bex1 belongs to a growing family of small proteins of unknown function, but with several features suggesting roles as adaptors or modulators of intracellular signaling pathways. Bex1 interacted directly with the intracellular domain of $p75^{NTR}$, but this interaction was not affected by NGF binding to the receptor, suggesting that Bex1 does not function as a downstream effector of $p75^{NTR}$ signaling, at least not in the conventional sense. On the other hand, the dynamic regulation of Bex1 levels during cell cycle, growth arrest and cell differentiation indicated that other signaling inputs or cellular states may regulate Bex1 binding to $p75^{NTR}$. Thus, for example, no Bex1 complexed with $p75^{NTR}$ could be recovered in cells synchronized in the G1 phase of the cycle by serum starvation. Together with the ability of Bex1 to modulate ligand-dependent recruitment of bonafide downstream effectors, such as the adaptor and serine–threonine kinase RIP2, these observations suggest that Bex1 may represent a novel class of upstream intracellular regulators of $p75^{NTR}$ function.

Bex1 showed a dynamic nucleocytoplasmic distribution in cells expressing $p75^{NTR}$ following stimulation with NGF, which resulted in rapid relocalization of Bex1 from the nucleus to the cytoplasm. This is also in contrast to several other known interactors of $p75^{NTR}$ which translocate to the nucleus upon NGF binding (Casademunt *et al*, 1999; Chittka and Chao, 1999). NGF could induce nuclear export of Bex1 in cells expressing $p75^{NTR}$ but not TrkA, suggesting a two-way communication between the interacting proteins, that is, $p75^{NTR}$ signaling regulates Bex1 localization, and Bex1 levels regulate $p75^{NTR}$ signaling. Phosphorylation of Ser-^{105} had no effect on the subcellular distribution of Bex1 (M Vilar, unpublished observations), so the mechanisms controlling nucleocytoplasmic trafficking of Bex1 remain to be elucidated. Intriguingly, although NGF induced a partial redistribution of Bex1 protein to the cytoplasm in all cell types tested, this did not translate into a detectable increase in its interaction with membrane-bound $p75^{NTR}$, perhaps due to the particular internalization kinetics of this receptor (Bronfman *et al*, 2003) or due to a distinct subcellular compartmentalization. Interestingly, the intracellular domain of $p75^{NTR}$ has been observed to undergo endoproteolytic cleavage and nuclear translocation (Jung *et al*, 2003; Kanning *et al*, 2003; Frade, 2005), although the studies reported so far differ as to the role of NGF in those processes.

In addition to $p75^{NTR}$ signaling, other stimuli are likely to regulate Bex1 function, as demonstrated by the ability of serum to induce Bex1 phosphorylation in Ser^{105} via the PI3K/AKT pathway. In the absence of serum, Bex1 was dephosphorylated—presumably due to low AKT activity—and rapidly degraded by the proteasome. The functional importance of the rapid Bex1 turnover is supported by at least two sets of

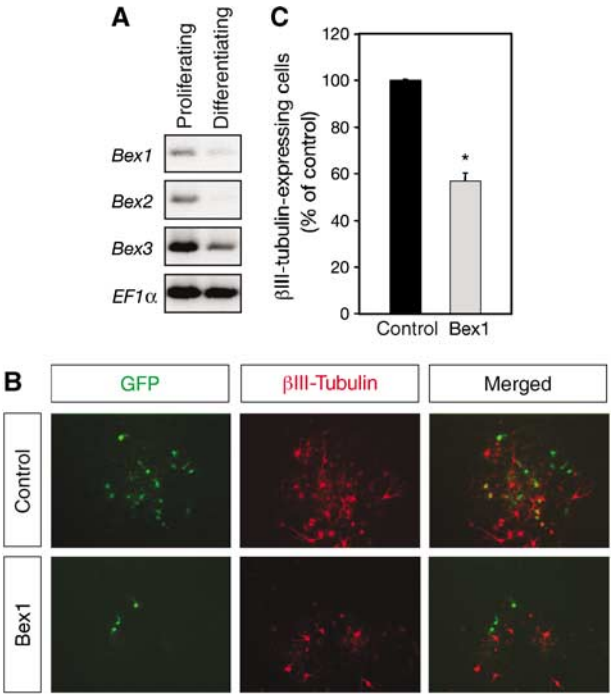

Figure 9 Bex1 inhibits neuronal differentiation of precursors from the brain SVZ. (**A**) *Bex1*, *Bex2* and *Bex3* mRNA levels were analyzed by RT–PCR in floating neurospheres derived from the postnatal SVZ (proliferating) or differentiating neural precursors after 5 days of mitogen withdrawal. *EF1α* is shown as loading control. (**B**) Neural precursors transfected with GFP (control) or GFP-Bex1 (Bex1) were allowed to differentiate after mitogen withdrawal. Micrographs show GFP fluorescence, βIII-tubulin immunocytochemistry (in red) and their overlay (merged). In the examples shown, double-positive cells (yellow) are observed in control, but not Bex1-transfected cells. (**C**) Quantification of neuronal differentiation of SVZ precursors as the percentage of βIII-tubulin-positive cells among the GFP-expressing cell population. Under control conditions (set here to 100%) 20–30% of the cells expressed βIII-tubulin and extended neurites. Average results of three independent experiments are shown ±s.d. $^*P<0.05$ versus control.

observations: (i) endogenous Bex1 levels oscillated during the cell cycle, being lowest at G1 and highest at S phase; (ii) preventing the normal cycling and downregulation of Bex1 had dramatic effects on cell proliferation and the ability of cells to exit the cell cycle and differentiate. The oscillation of Bex1 levels during the cell cycle could conceivably be controlled by cycles of phosphorylation and de-phosphorylation at Ser^{105}. However, whether AKT activity normally oscillates with the cycle in dividing cells has not yet been investigated. Likewise, following NGF addition, AKT only remains phosphorylated for 30–60 min, after which Bex1 may be dephosphorylated and degraded.

Bex1 overexpression caused PC12 cells to become resistant to growth arrest induced by serum withdrawal or NGF treatment, suggesting that Bex1 levels need to be downregulated at G1 in order for cells to exit the cycle and differentiate. In agreement with this, Bex1 knockdown accelerated cell cycle exit and neuronal differentiation. In PC12 cells, Bex1 overexpression interferred with cell cycle exit but not progression, suggesting that Bex1 is not an intrinsic component of the cell cycle machinery but may rather function as a gate-keeper of growth arrest and cell differentiation, preventing cell cycle exit during S, G2 and M phases. In contrast to Bex1, several previously identified downstream effectors of $p75^{NTR}$ have been shown to promote mitotic cycle arrest (Lopez-Sanchez and Frade, 2002; Chittka *et al*, 2004). Together with its ability to regulate $p75^{NTR}$ function, this suggests that Bex1 could be part of a feedback mechanism to gate $p75^{NTR}$ activity according to the phase of the cell cycle. In line with its effects in PC12 cells, Bex1 was downregulated upon differentiation of SVZ precursors, and its overexpression affected the ability of those cells to undergo neuronal differentiation upon mitogen withdrawal, indicating a broader role for Bex1 in the control of neuronal differentiation. Interestingly, neurospheres grown from the early postnatal SVZ have been shown to express $p75^{NTR}$, and lack of $p75^{NTR}$ expression was reported to decrease their capacity to undergo neuronal differentiation in response to BDNF (Hosomi *et al*, 2003).

The mechanisms by which Bex1 prevents cell cycle exit are at present unclear, but could involve interference with signaling pathways activated by growth arrest and differentiation signals. Despite the recognized importance of TrkA-derived signals in cell cycle exit and neuronal differentiation of PC12 cells, Bex1 overexpression did not interfere with the activation of two primary targets of TrkA signaling, that is, Erk1/2 and AKT. Additional studies will be required to establish whether Bex1 may affect further downstream steps of TrkA-activated pathways. On the other hand, elevated Bex1 expression prevented —and reduced expression enhanced— the induction of NF-κB activity by NGF. Upon NGF treatment, PC12 clones overexpressing Bex1 exhibited only very short neurites and failed to fully differentiate, a phenotype that resembled that obtained after inhibition of NF-κB activity in these cells (Foehr *et al*, 2000). In agreement with the importance of the interplay between Bex1 and RIP2 in this process, elevating RIP2 levels relieved the differentiation block of Bex1-overexpressing cells, suggesting that Bex1 may modulate $p75^{NTR}$ signaling by antagonizing the recruitment and activation of adaptor proteins such as RIP2.

In conclusion, our studies establish Bex1 as a novel link between the cell cycle and neurotrophic factor signaling. Unlike many other receptor-interacting molecules, Bex may function in part as an upstream modulator of receptor signaling, coordinating biological responses to external signals with internal cellular states. These findings open interesting possibilities for exploring Bex1 function in the control of neural stem cell renewal and neurogenesis in the peripheral and central nervous systems.

Materials and methods

T7 phage display

A T7 phage display cDNA library was prepared from differentiated PC12 cells according to manufacturer's instructions (Novagen). The intracellular domain of $p75^{NTR}$ ($p75^{ICD}$) was produced and purified as described previously (Liepinsh *et al*, 1997). Purified $p75^{ICD}$ was biotinylated using the EZ-link reagent (Pierce), and immobilized onto streptavidin-coated paramagnetic beads (Promega). Biopanning was performed using standard procedures (see Supplementary data).

RT–PCR and in situ hybridization

For RT–PCR studies, total RNA purification and first-strand cDNA synthesis were performed with kits from Invitrogen and Stratagene, respectively. Primer sequences for rat *Bex1*, *Bex2* and *Bex3* and RT–PCR conditions are available upon request. *In situ* hybridization was performed using standard protocols (see Supplementary data).

Cell culture and transfection

PC12, HEK293 T and Schwann cells were cultured using standard protocols (see Supplementary data). Synchronized PC12 cultures were obtained after 24 h incubation in serum-free medium. After serum re-addition, cell cycle phases were confirmed by flow cytometry. Cultures of SVZ-derived precursors were established from postnatal day 9 rat SVZ using standard protocols (see Supplementary data). Cell transfection of PC12, HEK293 T and Schwann cells was carried out using polyethylenimine (PEI, 25 kDa, Sigma-Aldrich) as described (Scott *et al*, 2005). Neural precursors were transfected using Fugene-6 (Roche) after one neurosphere passage, then allowed to grow in suspension for 5 days before induction of neuronal differentiation.

Immunoprecipitation, IB and metabolic labeling

Total cell lysates, immunoprecipitation (IP) and metabolic labeling were prepared and performed using standard protocols (see Supplementary data). Antibodies were obtained from various sources as indicated in the Supplementary data. Polyclonal anti-Bex1 antibodies were prepared by immunizing rabbits with a peptide derived from rat Bex1 (KNCAPRGGRRRFRVRQPI) conjugated to keyhole limpet hemocyanin.

Pharmacological treatments and siRNA

NGF (Alomone Labs) was used at 100 ng/ml in low serum containing medium (typically 1.5% FCS) unless otherwise indicated. Cycloheximide (Sigma) was used at 40 μg/ml, PS I (Calbiochem) at 25 μM and LY294002 (Calbiochem) at 50 μM. An siRNA construct targeting the rat *Bex1* mRNA sequence was assembled in the TOPO vector (Invitrogen) using the mouse U6 RNA Pol III promoter upstream of a hairpin DNA sequence derived from *Bex1* (see Supplementary data). An siRNA construct directed to *Wnt3* was used as negative control.

Flow cytometry, BrdU incorporation, cell death and gene reporter assays

Cell cycle phases were analyzed by flow cytometry in cells stained with propidium iodine as described previously (Rudkin *et al*, 1989; Urdiales *et al*, 1998). Cell proliferation was assessed by measuring BrdU incorporation using standard procedures (see Supplementary data). Cell death was quantified as the number of picnotic nuclei in cultures stained with DAPI. For cell differentiation assays, the proportion of cells bearing neurites longer than one- or two-cell diameters was quantified. Gene reporter assays were performed using standard protocols (see Supplementary data).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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SUPPLEMENTARY INFORMATION TO VILAR ET AL.

MATERIALS AND METHODS

T7 phage display

A T7 phage display cDNA library was prepared from differentiated PC12 cells according to manufacturer's instructions (Novagen). Briefly, poly(A) RNA was purified from differentiated PC12 cells 5 d after NGF treatment using a mRNA purification kit from Invitrogen. Randomly primed cDNA was synthesized, ligated with adapters, and unidirectionally cloned into the T7Select1-1b phage using the T7Select1-1 Cloning Kit according to the manufacturer's protocols (Novagen). The cDNA was size-selected by agarose gel electrophoresis to exclude fragments < 500 bp. The library was amplified once in liquid culture before use.

The intracellular domain of $p75^{NTR}$ ($p75^{ICD}$) was produced and purified as previously described (Liepinsh et al., 1997). Purified $p75^{ICD}$ was biotinylated using the EZ-link reagent from Pierce, and immobilized onto streptavidin-coated paramagnetic beads (Promega) by incubating 1 mg of beads with 50 mM biotinylated $p75^{ICD}$ in phosphate buffer saline (PBS) supplemented with 0.1% Nonidet P-40 (PBS-N) for 30 min at room temperature with gentle mixing. Beads were then washed four times in PBS-N, resuspended in 1 ml Superblock-PBS solution (Pierce) before biopanning.

Phage particles for biopanning were prepared by infecting *E. coli* BL5615 cells (Novagen) with $1x10^{10}$ p.f.u. of library stock. After complete lysis, the lysate was cleared by centrifugation and supplemented with *E. coli* protease inhibitor cocktail (Sigma-Aldrich, P-8465) and Pan Mix buffer (Novagen). Phages were then incubated with 0.1 ml beads coated with $p75^{ICD}$ during 30 min at room temperature or overnight at 4°C. Beads collected on a magnetic support were washed 4 times by complete resuspension in 1.5 ml PBS supplemented with 0.5% Nonidet P-40, and then eluted with 0.1 ml of 1% SDS for 15 min at room temperature. The eluate was used to infect BL5615 cells which were then plated

overnight until the appearance of plaques. Phages recovered by elution in PBS were supplemented with Pan Mix and used in a second round of biopanning. After a third round, serially diluted phage eluate was used to infect BL5615 cells for isolation of monoclonal phages. DNA inserts were amplified by PCR using primers described by the manufacturer, and analyzed by automatic DNA sequencing. Details of the complete T7 phage display protocol are available from the Novagen website at http://www.emdbiosciences.com/html/NVG/home.html. Micropanning experiments using the isolated Bex1 phage and unrelated controls confirmed the specificity of the interaction between this phage and the intracellular domain of $p75^{NTR}$ (data not shown).

Site-directed mutagenesis

Site-directed mutagenesis of various constructs was done using the Quick-Change method (Stratagene). All epitope taggings of Bex1 (HA, Flag, GFP and GST) were done at the N-terminus of the protein. GST-Bex1 was expressed in the pGEX1 vector (Promega), and produced and purified in *E. coli* following standard procedures.

Cell culture

PC12 cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal calf serum (FCS), and gentamycin, unless otherwise indicated. HEK293T cells were cultured in DMEM supplemented with 10% FCS and gentamycin. Schwann cells were extracted from the newborn rat sciatic nerve by collagenase treatment and expanded in DMEM supplemented with 10% FCS, 10 ng/ml basic FGF and 10 μM forskolin. Non-proliferative cultures were obtained by removing FGF and forskolin for 48 h. SVZ-derived neurospheres were grown in DMEM:F12 supplemented with B27 (Life Technologies), bFGF and EGF. After 5 days, they were dissociated, transfected, and allowed to grow in suspension for

another 5 days. Neuronal differentiation was initiated by plating on poly-D-Lysine-coated dishes in the absence of mitogens.

In situ hybridization

Rat brains from embryonic day (E) 13.5 and E19.5 were removed and rapidly frozen in OCT (optimal cutting temperature) compound at –70°C. In situ hybridization was performed on 14 µm cryosections with a 35S-labeled rat Bex1 riboprobe using standard procedures. Briefly, sections were first fixed in 4% PFA, deproteinized in 0.2 M HCl, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, and dehydrated in increasing concentrations of ethanol. Slides were incubated overnight in a humidified chamber at 53°C with 106 cpm of probe in 200 µl of hybridization cocktail, washed, dehydrated, air-dried, and dipped in NTB-2 photoemulsion (Kodak). After 8 week exposure, slides were developed and counterstained with thionin.

Immunoprecipitation, immunoblotting and metabolic labeling

Total cell lysates for immunoprecipitation were obtained by extracting cell monolayers with 1% Triton X-100, 60 mM β-octylglucoside, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA supplemented with a protease inhibitor cocktail (Roche) for 60 min at 4°C. For rapid cell lysis that did not require subsequent immunoprecipitation, cell monolayers were directly lysed in 10% trichloroacetic acid (TCA), centrifuged and boiled in SDS-PAGE sample buffer. Immunoprecipitations were performed by overnight incubation at 4°C with the corresponding antibodies followed by precipitation with gamma-bind protein-G beads (Amersham), and elution by boiling in sample buffer. SDS-PAGE and immunoblotting were performed using standard procedures. After processing, PVDF filters were developed by Enhanced Chemifluorescence (ECF, Amersham) and scanned in a STORM 840 fluorimager (Molecular Dynamics). For metabolic labeling, cell monolayers were pre-incubated in phosphate-free

DMEM supplemented with dialyzed FCS, followed by 1 h incubation with 1 mCi/ml of ^{32}P orthophosphate in phosphate-free DMEM. After washing with PBS, cells were lysed as above.

Antibodies

Antibodies were obtained from various sources as follows: anti-phospho-ERK (Thr-202/Tyr-204), anti-AKT, anti-phospho-AKT and anti-phospho-AKT substrate from New England Biolabs; monoclonal anti-HA from Covance; anti-Flag, anti-actin and anti-α-tubulin from Sigma; anti-myc from Santa Cruz; anti-p75^{NTR} extracellular domain MC192 mouse monoclonal from Chemicon; anti-GST, secondary anti-mouse and anti-rabbit IgG conjugated to peroxidase from Amersham. Rabbit polyclonal anti-serum against the intracellular domain of p75^{NTR} (9992) was kindly provided by Dr. Moses Chao.

BrdU incorporation and gene reporter assays

BrdU was used at 10 μM for 30 min or 5 h. Anti-BrdU monoclonal antibody was from DAKO. BrdU positive nuclei were counted under epifluorescence illumination and expressed as the percentage of the total number of nuclei by counter-staining with DAPI. For gene reporter assays of NF-κB activity, cells were transfected with a plasmid carrying the firefly luciferase gene under the control of a promoter sensitive to NF-κB (Clontech) and the Renilla luciferase plasmid pRL-TK (Promega) to control transfection efficiency. One day after transfection, cultures were treated with 100 ng/ml NGF for 4 h and processed with the Dual-Luciferase Reporter Assay kit from Promega. The values obtained for firefly luciferase activity in each well were normalized with the corresponding values of Renilla luciferase activity.

siRNA and Bex1 knock-down

The hairpin DNA sequence used in the Bex1 siRNA construct was as follows: 5'-GCAAAAATTGTGCGCCTAGAGTCTCTTGAACTCTAGGCGCACAATTTTTGC-3',

with the loop region underlined. The following protocol was used to assess the effects of the Bex1 siRNA construct on neuronal differentiation. Forty eight hours after transfection with the siRNA plasmid construct, PC12 cells were synchronized by 24 h serum starvation. Serum was then replaced (i.e. 72 h after transfection) to re-initiate the cell cycle, and neuronal differentiation was induced 12 h later —i.e. during the S phase of the cell cycle— by addition of 100 ng/ml NGF. Neuronal differentiation was then assessed at 12, 36 and 60 h following NGF addition by scoring the proportion of cells bearing neurites longer than 1- or 2-cell diameters.

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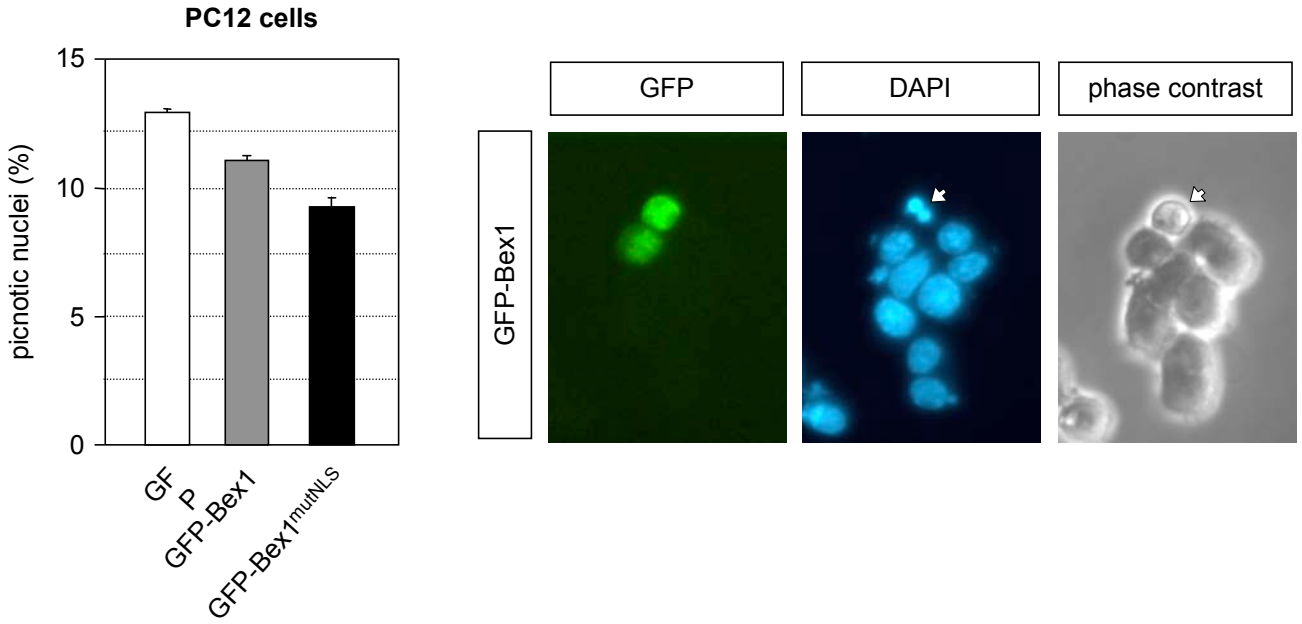
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Bex1 overexpression does not affect PC12 cell survival.

The percentage of picnotic nuclei were quantified in PC12 cells 24 h after transient transfection with the indicated plasmid combinations. Shown are averages of three independent experiments (each performed in triplicate) ± SEM. Micrographs to the right show two cells transfected with GFP-Bex1 (GFP panel), one of which showed a picnotic nucleus (arrow, DAPI panel).

Supplementary Figure 2.

Flow cytometry analysis of serum-starved cultures of parental PC12 cells and clone C4 overexpressing Bex1. Cell cycle phases are indicated. Note the enlarged proportion of cells in S phase in clone C4 compared to parental cells.


PC12 cells
picnotic nuclei (%)
15
10
5
0
GFP
GFP-Bex1
GFP-Bex1mutNLS
GFP
DAPI
phase contrast
GFP-Bex1

24h serum free